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Prognostic Value of Protein Kinase C, Proto-Oncogene Products and Resistance-Related Proteins in Newly Diagnosed Childhood Acute Lymphoblastic Leukemia

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In this investigation, untreated non-B-type acute lymphoblastic leukemia (ALL) of 104 children was analyzed using immunocytochemistry for expression of protein kinase C, proto-oncogene products (Fos, Jun, Ras) and resistance-related proteins (topoisomerase II, P-glycoprotein, glutathione S-transferase-π, metallothionein, dihydrofolate-reductase, thymidylate-synthase). The aim of the analysis was to find out whether combining those factors with the most important clinical prognostic factor (blast cell count) can improve the prognostic value (relapse-free interval). Univariate analysis shows that protein kinase D (PKC), Fos, P-glycoprotein (P-170) and glutathione S-transferase-π (GST-π) are significant prognostic factors independent of blast cell count (PBC) for the relapse-free intervals of children with ALL. The presence of the proteins Fos, PKC, P-170 and GST-π was not independent within the patient population. The multivariate analysis showed that in combination with PBC and PKC, both P-170 and GST-π have only limited prognostic influence. Combining the factors PKC, Fos and GST-π as a categorial variable showed that this variable is a strong prognostic factor in addition to PBC. **Med. Pediatr. Oncol. 28:117–126**

Key words: prognosis; proto-oncogenes; protein kinase C; resistance-related proteins; immunocytochemistry

INTRODUCTION

A number of baseline characteristics (e.g., blast cell counts in peripheral blood) have been used to predict outcome in patients with acute lymphoblastic leukemic cells after treatment. In recent years, based on experimental studies in the laboratory at the cellular and molecular levels, there have been a number of studies examining the prognostic ability of newer prognostic factors.

There is increasing evidence that in tumor cells a wide variety of drug resistance mechanisms are present which are responsible for the outcome of patients after treatment. For instance, resistance to a broad range of structurally different drugs is correlated with the overexpression of a membrane glycoprotein which has been termed P-glycoprotein (P-170) [1–6]. However, not all resistant tumor cells express P-170, so that refractoriness to chemotherapy can only partly be explained by the expression of this protein. This suggests that other mechanisms are also implicated in the acquisition of resistance. Glutathione S-transferases are isoenzymes which conjugate glutathione with various xenobiotics [7]. These proteins may therefore play an important role in the detoxification of drugs such as cyclophosphamide and anthracyclines, which are involved in leukemia therapy. In addition, a number of antineoplastic drugs, such as intercalating anthracyclines, affect topoisomerase II [8]. Metallothionein was also found to be involved in drug resistance [9]. Several reports show that resistance against cisplatin is mediated by an overexpression of metallothionein [10]. Tumor cells resistant to cisplatin and adriamycin display increased levels of thymidylate-synthase [11,12]. In experimental models but also in human tumors several mechanisms of methotrexate (MTX) resistance have been identified [13]. The most common mechanism of MTX-resistant cells developed in vivo and MTX-resistant cells isolated from clinical samples had increases in dihydrofolate-reductase (DHFR).

There are now various reports which suggest that cell populations exist in human tumors which have several resistance mechanisms operative at once [14]. Recently, we demonstrated an increased expression of P-glycoprotein not only with concomitant overexpression of glutathione S-transferase-π, but also with coordinate overexpression of metallothionein and thymidylate-synthase in human lung tumors [15–17]. Although our knowledge is limited as to which factors are responsible for a regulated coexpression of resistance mechanisms, one possibility is that the resistance factors belong to a set of genes which is controlled by general regulatory mechanisms

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[18]. The c-fos/c-jun protein complex, which binds specifically to AP-1, affects the transcriptional expression of several cellular genes and, interestingly, P-glycoprotein, glutathione S-tranferase and metallothionein contain an AP-1 motif [19,20]. Thus, these genes may be regulated by the proto-oncogenes c-fos and c-jun. In addition, nuclear protein kinase C is of high functional importance as a stimulator of the activity of proto-oncogenes such as c-fos and c-jun. Thus, a possible involvement of second messenger systems and their related enzymes in the development of resistance has also been discussed [21].

In the present study, we analyzed whether combining the products of resistance-related genes, the products of proto-oncogenes, and protein kinase C with the most important clinical prognostic factor, namely the blast cell count, can improve the prognostic value in children with acute lymphoblastic leukemia (ALL). For this reason, untreated non-B-type ALL of 104 children was analyzed using immunocytochemistry for expression of the proto-oncogene products Fos, Jun, Ras, the protein kinase C (PKC), and the resistance-related proteins topoisomerase II, P-glycoprotein, glutathione S-transferase-π, metallothionein, dihydrofolate-reductase and thymidylate-synthase.

MATERIALS AND METHODS

Patients

One hundred and four children with newly diagnosed non-B-type ALL were investigated. The criterion for patient selection was the availability of cell probes. All patients with available cells were enrolled in this retrospective study. For this reason, patients with high initial blast cell counts (frequently T-ALLs) are more present in this group. The diagnosis of leukemia was made by standard cytological and histochemical examination of bone marrow and blood smears according to the French-American-British (FAB) classification [22] and by immunological investigation of the blast cells using indirect immunofluorescence. Patients were divided into three subgroups: (a) precursor B-ALL (HLA-DR, CD 19), (b) common (c)-ALL (HLA-DR, CD 10, CD 19) and (c) T-ALL (CD 1, CD 2, CD 7). The CD 13, CD 33 and CD 65 antigens were examined in order to define myeloid markers. Eligible for the study were patients who entered complete remission after standardized treatment [23,24]. Complete remission was diagnosed if the blast cell content was less than 5% in an otherwise normocellular marrow on day 33 after the onset of the therapy without evidence of blast cells at extramedullary sites. Not included were seven patients who died before the onset of chemotherapy. The patient's characteristics are given in Table I.

TABLE I. Patient Characteristics and Median Relapse-free Intervals (MRFI)

		Patients (n)	MRFI (years)	*P*-value (log-rank test)
Age (years)	0–9	80	>4	NS[c] (0.51)
	≥10	24	>4	
Sex	Male	46	>4	NS (0.49)
	Female	58	>4	
FAB-type[d]	L1	76	>4	NS (0.18)
	L2	28	2.1	
Immunological subtype[a]	pre-B-All	17	>4	
	C-ALL	47	>4	NS (0.41)
	T-ALL	33	2.2	
PBC [10^9/l][b]	<50	61	>4	
	≥50	43	1.4	0.01
Therapy	A	66	>4	
	B	22	>4	NS (0.11)
	C	16	>4	

[a]Seven patients were not available for immunotyping.
[b]PBC, peripheral blast cell count.
[c]NS, not significant.
[d]French-American-British (FAB) classification.

Treatment

All 104 patients received treatment according to the protocols (ALL-VII/81, ALL-VIII/87 [23] and ALL-BFM/90 [24]. These treatment protocols consist of induction therapy with prednisone, vincristine, daunorubicin and L-asparaginase followed by consolidation therapy with cyclophosphamide, cytarabine, 6-mercaptopurine and MTX. Patients included in study ALL-VII/81 (study A, n = 66) and ALL-VIII/87 (study B, n = 22) received intermediate-dose intravenously MTX (0.5 or 1 g/m^{-2}); patients included in study ALL-BFM/90 (study C, n = 16) received high-dose MTX (5 g/m^{-2}). Maintenance therapy was performed with oral 6-mercaptopurine (daily) and methotrexate (weekly) for up to 2 years after starting therapy. The treatment results of the patients included in our study were statistically not different concerning the three treatment protocols ($P = 0{,}11$). Therefore, all patients of the three groups were used for the prognostic evaluations.

Leukemic Cells

Cell samples were collected in heparinized flasks and mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. After being washed twice in culture medium (RPMI 1640), the cells were cryopreserved in liquid nitrogen with 10% dimethylsulphoxide and 5% fetal calf serum using a programmed freezer. All samples contained at least 80% blast cells (examined by May-Grunwald-Giemas staining).

Immunocytochemistry

In order to measure the proteins, cell samples were resuspended in Hanks' balanced salt solution (Biochrom, Berlin, Germany) and the viability of cells was tested by staining with methylene blue. Cell suspensions were centrifuged with a Cytospin II (Shandon, Frankfurt, Germany), resulting in a cell monolayer. After air drying, the cells were fixed in ice-cold acetone for 10 min and stored at −20°C. Immunohistochemical investigations were performed using the streptavidin-biotin peroxidase-complex method [25,26]. Cell preparations were briefly preincubated with hydrogen peroxide (0.3%; 15 min), unlabelled streptavidin (dilution 1:50; 15 min) and nonimmune normal serum. For detection of PKC, we used the monoclonal antibody MC5 (Amersham, Braunschweig, Germany) in a working concentration of 10 μg/ml. This antibody recognizes the α- and β-form but not the γ-form of PKC. For detection of the protein of the protooncogene c-fos, the rabbit polyclonal antibody c-fos (Ab-2, Dianova, Hamburg, Germany) was used. This antibody was developed against a peptide corresponding to residues 4–17 of human Fos [27]. For the determination of the c-jun product, the rabbit polyclonal antibody c-jun/AP-1 (Ab-1, Dianova, Hamburg, Germany) was used. This antibody was developed against a peptide corresponding to the amino acids 209–225 of v-Jun. For immunostaining of the pan-ras product, the mouse monoclonal antibody pan-ras (Ab-1, Dianova) was applied. For detection of topoisomerase II, a polyclonal antibody (topoGen; Columbus OH) was used. This antibody recognizes the 170-kDa form (topoisomerase II). For the detection of P-glycoprotein, the murine monoclonal antibody C219 (Isotopen-Diagnostic, Dreieich, Germany) with specificity to an internal epitope was used. The final concentration was 10 μg/ml. A rabbit polyclonal antibody (GST-π, dilution 1:2,000; kindly provided by Dr. K. Satoh, University School of Medicine, Hirosaki, Japan) was used for the detection of GST-π. The anti-human thymidylate-synthase antibody was a kind gift from Dr. B. Yates (Burroughs Wellcome, Research Triangle Park, NC). It was used in a working dilution of 1:500. The anti-dihydrofolate-reductase antibody was kindly provided by Dr. J. H. Freisheim (Medical College of Ohio, Toledo, OH). The working dilution of the antibody was 1:250. For detection of metallothionein (MT), the mouse monoclonal anti-MT-antibody (DAKO-MT, E9; DAKO-Diagnostika, Hamburg, Germany; dilution 1:100) was used. The primary antibodies were applied for 16 h at 4°C in a moist chamber. After three washes in phosphate-buffered saline (PBS), the cells were incubated for 30 min with biotinylated sheep anti-mouse IgG (Amersham, Braunschweig, Germany) or with goat anti-rabbit IgG (Dianova), both diluted 1:50 with 5% normal human serum. Afterwards, the streptavidin-biotinylated peroxidase complex (Amersham; 1:100, 30 min) was added. Peroxidase activity was made visible with 3-amino-9-ethylcarbazole (15 min), which gives a red-brown reacting product. Counterstaining was performed with haematoxylin and the sections were mounted with glycerol gelatine. Negative controls were obtained firstly by omitting the primary antibodies and secondly by an irrelevant antibody.

Three observers independently evaluated and interpreted the results of immunocytochemical staining. The immunocytochemical staining was graded either as negative (=0), weakly positive (=1), moderately positive (=2), or strongly positive (=3) according to a score which we had previously validated in a series of animal as well as human cell lines and human solid tumors. The immunostaining was scored without knowledge of the diagnosis or other clinical parameters. The evaluations were in concordance in 90% of patients. The other patients (10%) were reevaluated independently and then classified according to the classification given most frequently by the observers.

Statistical Analysis

Life table analyses according to Kaplan and Meier [28] were performed for relapse-free intervals. Patients who died in remission were used as censored cases. The groups were compared by log-rank tests. The prognostic influence of clinical and molecular parameters was assessed by multivariate regression methods (Cox model) as described by Byar [29]. The interrelationships of clinical data and molecular parameters were assessed statistically by using Fisher's exact test [30], which was used as a statistical hypothesis test for the presence or absence of an association between two factors. For the analysis, the different factors were classified as negative (neg) or positive (pos). Leukemic cells were classified as negative when there was complete absence of staining for Fos, Jun, Ras, PKC, P-glycoprotein and metallothionein (score = 0) and positive when those factors had a score of 1–3. Leukemic cells were graded as glutathione S-transferase-π-negative, thymidylate-synthase-negative, topoisomerase-negative, and dihydrofolate-reductase-negative when there was complete absence of staining (score 0) or weak staining (score 1) originating of baseline expression. In these cases leukemic cells with 2 and 3 scores were classified as positive. The immunohistologic staining was expressed according to a score that we have validated in a series of animal and human cell lines and human solid tumors. In earlier investigations, the immunohistologic staining was also validated by quantification using radioimmunoassay measurements. With respect to the blast cell counts the patients were also grouped in two collectives: low blast cell count ($<$50,000 mm^3 = negative) and high blast cell count ($\geq$50,000 mm^3 = positive) as it is commonly used for clinical prognosis. Dichotomization of peripheral blast cell count

TABLE II. Protein Kinase C, Products of Proto-Oncogenes (Fos, Jun, Ras) and Resistance-Related Proteins and Median Relapse-Free Intervals (MRFI) of Children With ALL

		Patients (n)	MRFI (years)	*P*-value (log-rank-test)	*P*-value (bivariate Cox regression with PBC)[c]
PKC[c]	Negative	56	>4		
	Positive	48	2.2	0.004	0.005
Fos	Negative	52	>4		
	Positive	52	2.2	0.0009	0.005
Jun	Negative	39	>4		
	Positive	65	>4	NS[b] (0.11)	
Ras (H)	Negative	82	>4		
	Positive	22	>4	NS (0.85)	
Topo[a,c]	Negative	44	>4		
	Positive	37	>4	NS (0.33)	
P-170[c]	Negative	68	>4		
	Positive	36	2.9	0.025	0.031
GST[a,c]	Negative	52	>4		
	Positive	52	2.9	0.027	0.073
MT[a,c]	Negative	58	>4		
	Positive	28	2.9	NS (0.14)	
DHFR[a,c]	Negative	71	>4		
	Positive	25	>4	NS (0.53)	
TS[a,c]	Negative	53	>4		
	Positive	49	>4	NS (0.87)	

[a]Tumor material was not available for all determinations.
[b]NS, not significant (*P*-value in parentheses).
[c]PKC, protein kinase C; Topo, topoisomerase II; P-170, P-glycoprotein; GST, glutathione S-transferase-π; MT, metallothionein; DHFR, dihydrofolate-reductase; TS, thymidylate-synthase.

(PBC) at the cutpoint of 50,000 blast cells/mm^3 was confirmed by specific statistical methods (residuals and maximally selected statistics). Prognostic significance of the dichotomized version of PBC was decreased in comparison with the continuous version both in the univariate analysis ($P = 0.01$ vs. $P = 0.004$) as well as in the multivariate analyses when combined with the four factors PKC, Fos, P-170 and GST ($0.012 \leq P \leq 0.051$ vs. $0.0002 \leq P \leq 0.005$), each. Dichotomization of PBC and its decrease of significance had almost no influence on the prognostic significance of the other four factors and a use of the continuous version of PBC would not have decreased their significance; e.g., in the bivariate analysis (see Table II) the significance of both PKC and Fos would have changed from $P = 0.005$ to $P = 0.007$ and the significance of P-170 and GST would have changed from $P = 0.03$ to $P = 0.02$ and from $P = 0.07$ to $P = 0.08$, respectively, when using the continuous instead of the dichotomized version. Therefore, because of clinical convenience we mainly present here the results obtained with the dichotomized PBC.

RESULTS

The aim of the analysis was to find out whether combining expression of protein kinase C, proto-oncoproteins and resistance-related proteins with the most important clinical prognostic factor (blast cell count) can improve the prognosis of the children. In Table I the patients' data are given. Figure 1 shows expression patterns of protein kinase C (Fig. 1a), Fos (Fig. 1b), Jun (Fig. 1c), Ras (Fig. 1d), topoisomerase II (Fig. 1f), P-glycoprotein (Fig. 1g), glutathione S-transferase (Fig. 1h), metallothionein (Fig. 1i), thymidylate-synthase (Fig. 1j) and dihydrofolate-reductase (Fig. 1k). Figure 1e and l reveals negative controls. The protein kinase expression was detectable at the cell membrane (Fig. 1a). Fos and Jun were expressed in the nucleus (Fig. 1b,c) and Ras at the cell membrane (Fig. 1d). Topoisomerase II was detectable as homogenous coloration of the nuclei (Fig. 1f). P-170 staining was seen as typical membrane coloration (Fig. 1g) and glutathione S-transferase staining was found homogeneously distributed in the cytoplasm (Fig. 1h). Metallothionein, dihydrofolate-reductase and thymidylate-synthase were stained in the cytoplasma (Fig. 1c,j,k).

The prognosis of children with ALL is largely determined by the initial PBC. This is also true in our patients' group. Patients with PBC of 50,000 mm^{-3} or more tended to have more relapses than patients with PBC <50,000 mm^{-3} (data not shown). The median relapse-free interval was 1.4 years in patients with PBC of 50,000 mm^{-3} or more (positive) and >4 years in patients with PBC <50,000 mm^{-3} (negative). This difference is statistically significant (Table I, $P = 0.01$, log-rank test). The probability of remaining in first continuous complete remission

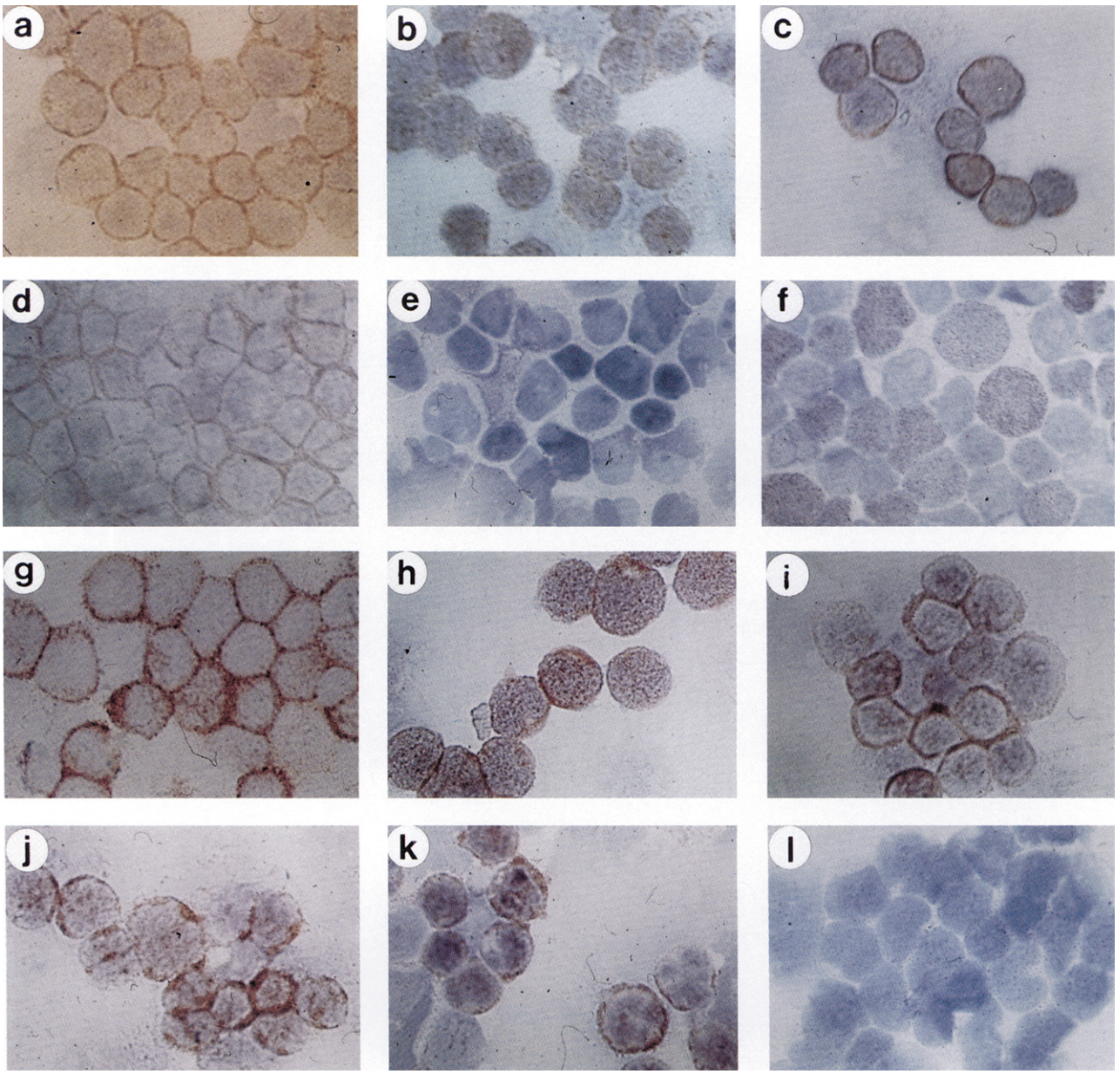

Fig. 1. Typical immunocytochemical staining of the proteins **a,** proteins kinase C; **b,** Fos; **c,** Jun; **d,** Ras; **e,** negative control; **f,** Topoisomerase II; **g,** P-glycoprotein; **h,** glutathione S-transferase-π, **i,** metallothionein; **j,** thymidylate-synthase; **k,** dihydrofolate-reductase; **l,** negative control.

is presented in Figure 2. Age, sex, Fab-type, immunological subtypes and treatment had no significant effect on relapse-free intervals (Table I).

In order to discover further cellular prognostic factors additional to blast cell count, the expression of PKC was analyzed immunocytochemically (Fig. 1a). Of the 104 cases of ALL, 56 (54%) showed no expression of PKC; 48 (46%) showed positive staining (Table II). In our analysis, the probability of remaining in first remission of patients with PKC-negative leukemic cells was significantly higher than in PKC-positive leukemias ($P = 0.004$, log-rank test). There is no interrelationship between the blast cell count and the expression of PKC (Table III). The results of the multivariate analysis demonstrate that PKC expression is a significant prognostic factor in addition to the peripheral blast cell count (Table II; PKC: $P = 0.005$; PBC: $P = 0.012$; Cox regression model). To determine whether the combination of peripheral blast cell and expression of PKC has a higher prognostic significance, the patients were grouped on the basis of blast

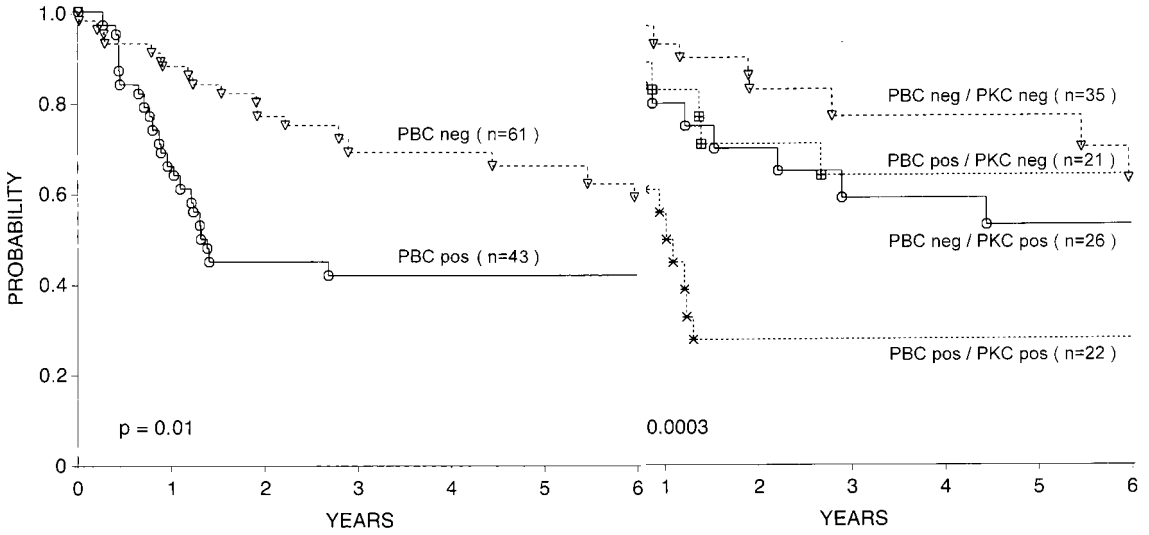

Fig. 2. Kaplan-Meier estimates of the relapse-free interval in children with low blast cell count (PBC neg; $< 50{,}000$ mm^3) and with high blast cell count (PBC pos; $\geq 50{,}000$ mm^3).

Fig. 3. Kaplan-Meier estimates of the relapse-free interval in children with low blast cell count and PKC-negative ALL (PBC neg/PKC neg), with high blast cell count and PKC-negative ALL (PBC pos/PKC neg), with low blast cell count and PKC-positive ALL (PBC neg/PKC pos) and with high blast cell count and PKC-positive ALL (PBC pos/PKC pos).

TABLE III. Interrelationships Between Blast Cell Count (PBC), Protein Kinase C (PKC) Fos, P-Glycoprotein (P-170) and Glutathione S-Transferase-π GST)

		PBC		*P*-value
		<50,000	≥50,000	(Fisher's exact test)
PKC	Negative	31	21	NS[a] (0.78)
	Positive	30	22	
Fos	Negative	36	25	NS (0.24)
	Positive	25	27	
P-170	Negative	41	27	NS (0.64)
	Positive	20	16	
GST	Negative	37	25	NS (0.80)
	Positive	23	14	

[a]NS, not significant (*P*-value in parentheses).

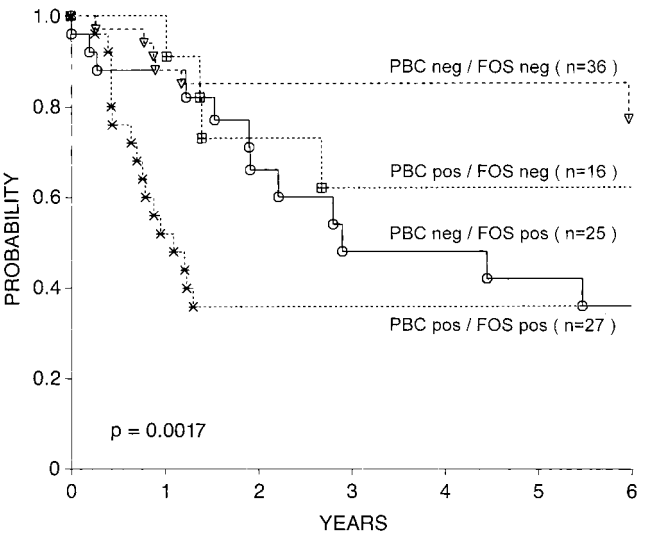

Fig. 4. Kaplan-Meier estimates of the relapse free intervals in children with ALL. Blast cell counts were combined with Fos expression.

cell counts and expression of PKC. Figure 3 shows that the prognosis of the patients according to the probability of remaining in first complete continuous remission is more distinguishable by combining both factors.

The expression of the proto-oncogenes c-fos, c-jun and c-ras were also analyzed immunocytochemically. In Figure 1, expression patterns of the oncogene products c-fos, c-jun and c-ras are shown. Of the ALL, 50% were positive for Fos (52/104), 63% for Jun (65/104) and 21% for Ras (22/104; Table II). The median relapse-free intervals of the patients grouped according to the expression of the oncoproteins are shown in Table II. The relapse-free interval was significantly shorter in patients with Fos-positive leukemic cells than in patients with Fos-negative leukemic cells ($P = 0.0009$, log-rank test). The expression of Jun and Ras in leukemic cells showed no significant correlation with the relapse-free intervals of the patients. Expression of Fos was independent of the PBC (Table III). The results of the multivariate analysis (Cox regression model) clearly demonstrate that both Fos and PBC are significant prognostic factors for the relapse-free intervals (Fos: $P = 0.005$; PBC: $P = 0.051$). Combining Fos expression and blast cell count showed that the prognostic value was improved (Fig. 4).

The expression patterns of the resistance-related pro-

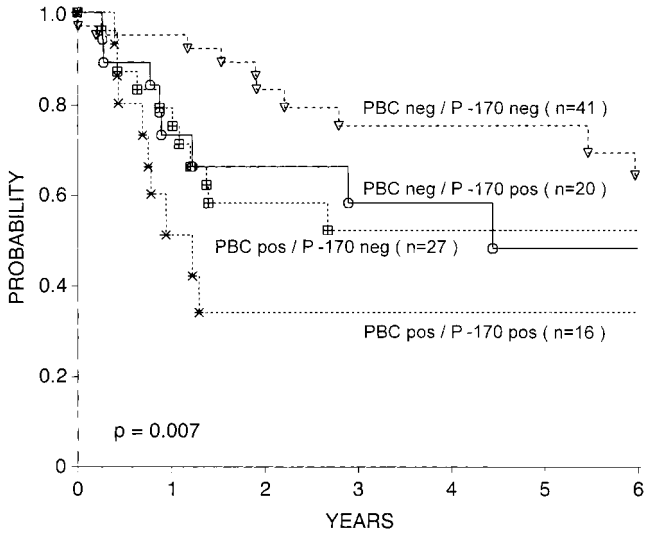

Fig. 5. Kaplain-Meier estimates of the relapse-free intervals in children with ALL. Blast cell counts were combined with P-glycoprotein (P-170) expression.

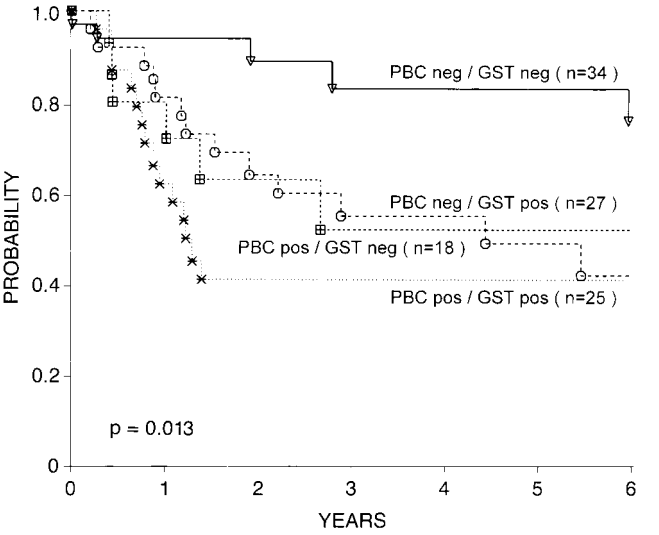

Fig. 6. Kaplan-Meier estimates of the relapse-free intervals in children with ALL. Blast cell counts were combined with glutathione S-transferase-π (GST).

teins are given in Figure 1 and Table II. High topoisomerase II expression was found in 46% (37/81) of ALL. But no differences were seen in disease-free survival of patients with low or high expression of topoisomerase II (Topo-negative vs. Topo-positive; $P = 0.33$). P-glycoprotein (P-170) expression was found in 36 out of 104 leukemias (35%), while 68 patients (65%) failed to express P-170 in the leukemic cells (Table II). Patients with P-170-positive cells had significantly lower median relapse-free intervals ($P = 0.025$; log-rank test). There is no interrelationship between expression of P-170 and blast cell count (Table III). The prognostic impact of P-170 in the presence of clinical data (PBC) was significant. An analysis of the reduced model with only PBC and P-170 as covariates resulted in a significant influence of both PBC ($P = 0.014$) and P-170 ($P = 0.031$). Figure 5 shows the results when both factors (PBC, P-170) are combined. Overexpression of glutathione S-transferase (GST) was present in 52 of the 104 cases (Table II). The median relapse-free intervals for GST-positive patients were significantly shorter than for GST-negative patients ($P = 0.027$; log-rank test). There was no interrelationship between blast cell count and expression of GST-π (Table III). A multivariate analysis showed that a significant influence of PBC exists ($P = 0.031$), whereas GST-π was borderline significant ($P = 0.073$). Figure 6 shows that, nevertheless, the prognostic value of the patients according to the probability of remaining in first complete continuous remission is improved by combining both factors.

In the present analysis, 86 ALLs were investigated for the expression of metallothionein (MT; Table II). Expression of MT was found in 28 (33%) of the cases. The median relapse-free survival tended to be lower in the MT-positive group, but this finding was not statistically significant ($P = 0.14$; log-rank test). Figure 1 and Table II also show the expression pattern of dihydrofolate-reductase (DHFR) and thymidylate-synthase (TS). Of the 96 leukemias investigated, 25 (26%) were DHFR-positive. No differences were seen in disease-free survival of patients with DHFR-negative and DHFR-positive ALL (Table II). Of the 102 ALL investigated, expression of thymidylate-synthase (TS) was seen in 49 cases (48%). The disease-free survival was not different in patients with TS-negative and TS-positive leukemia (Table II).

In conclusion, the results demonstrate that blast cell count, protein kinase C, Fos, P-glycoprotein and glutathione S-transferase-π in ALL are significantly linked with the relapse-free intervals of the children, whereas the proto-oncogene products Jun and Ras, and the resistance-related proteins topoisomerase II, dihydrofolate-reductase, thymidylate-synthase and metallothionein showed no relationships with the relapse-free intervals of the patients. Protein kinase C, Fos, P-glycoprotein and glutathione S-transferase are significant prognostic factors in addition to clinical prognostic factors (blast cell count) and can improve the prognostic value for the relapse-free intervals of children with ALL.

However, the presence of resistance-related proteins was not independent within the patients' population examined here. For instance, positiveness with respect to P-170 and GST was strongly correlated with positiveness of PKC ($P < 10^{-6}$ and $P = 0.006$, χ^2-test). However, P-170 and GST were not significantly correlated ($P = 0.09$, χ^2-test). The presence of Fos was strongly correlated with positiveness with respect to PKC ($P < 10^{-4}$, χ^2-test) and GST ($P < 10^{-4}$, χ^2-test) but not so strong to P-170

($P = 0.03$, χ^2-test). Finally, a multivariate analysis of the prognostic power of the factors PKC, Fos, P-170 and GST was performed in a sequence of steps. First, we analyzed each factor together with PBC for their respective influence on relapse-free survival as just described and shown in Table II (sixth column). A straightforward multivariate Cox regression of the four factors PKC, Fos, P-170 and GST together with PBC and a step-up or step-down model selection was prohibited from the above-stated correlations between some of the four factors. Since PKC appeared as the most important factor in a number of subanalyses (results not shown) in a second step, this factor was chosen as stratifying variable, and guided by the existing correlations we performed two separate analyses: (1) PBC together with GST and P-170; and (2) PBC together with Fos, both separately for the strata PKC = negative and PKC = positive.

There was no significant effect of GST and P-170 in the subgroups of patients with PKC = negative and even the effect of PBC was not significant ($P = 0.61$), whereas in the subgroup of PKC = positive the effect of PBC was statistically significant ($P = 0.02$). This shows that in combination with PBC and PKC, both GST and P-170 have no separate prognostic influence. The situation was qualitatively different when PBC and Fos were combined. When PKC = negative, Fos showed some prognostic effect ($P = 0.09$) and PBC none ($P = 0.56$). But when PKC = positive, only PBC showed a significant effect ($P = 0.04$) and no more Fos. Again, we see some prognostic influence of PBC and of Fos in at least one of the two PKC strata, separately. When PBC was not included in the multivariate model the qualitative results of PKC, GST, P-170 and Fos remained the same. In a third and final step of the multivariate analysis, we tried to synthesize the evaluation by combining the variables. This was achieved by using a synthesized factor PFG = PKC + Fos + GST as a categorical variable. This synthesized factor appeared as a stronger prognostic factor ($P = 0.031$) than PBC ($P = 0.48$; Table IV). P-170 showed in this combination no significant prognostic value ($P = 0.6$). A final model without P-170 (see Table IVb) confirmed the prognostic predictability of the synthesized factor PFG ($P = 0.015$) independent of the influence of PBC ($P = 0.052$).

TABLE IV. Multivariate Analysis of Prognosis of Relapse-Free Intervals Using a Synthetic Factor Combining PKC, Fos and GST Together With P-170 and PBC (n = 104)

		Relative risk estimate	*P*-value
(a)	PBC ≥50,000	1.90	0.048
	P-170-positive	1.21	0.60
	PKC/Fos/GST (PFG)		0.031
	One factor positive	2.32	
	Two factors positive	2.98	
	Three factors positive	4.74	
(b)	Without P-170		
	PBC ≥50,000	1.87	0.052
	PKC/Fos/GST (PFG)		0.015
	One factor positive	2.43	
	Two factors positive	3.15	
	Three factors positive	5.37	

DISCUSSION

An accurate prediction of relapse-free intervals of children with acute lymphoblastic leukemia remains a major problem in cancer therapy. Therefore, we compared the presence of resistance-related proteins with the clinical outcome. As response criteria we used the relapse-free interval. We found a significantly lower probability of remaining in first continuous complete remission in patients with *P*-glycoprotein (P-170)-positive or glutathione S-transferase-π-positive (GST-π) blast cells. Both parameters were independent of the blast cell count, the most important clinical prognostic factor. By combining P-170 and GST-π with the blast cell count, the prognostic significance with respect to the relapse-free interval was improved [6]. Musto et al. [31] reported a high risk of early relapse in leukemia patients with detectable P-170-positive cells in complete remission. Similar results were published on adult ALL on the basis of MDR-mRNA detection [32,33]. Schisselbauer et al. [34] also found detectable GST-π levels and a quantitatively increased GST activity in chlorambucil-resistant chronic lymphocytic leukemia (CLL) patients.

Several studies showed that topoisomerase II levels are low in leukemic cells [35,36]. The authors suggested that the resistance to doxorubicin is due to the extremely low levels of topoisomerase II in these cells. In general, reports about the activity of topoisomerase II in ALL are rare. Gekeler et al. [35] found no significant correlation between topoisomerase II mRNA expression and responsiveness to chemotherapy in ALL blast cells. In agreement with these studies we did not observe a significant correlation between topoisomerase II expression and the clinical outcome either. This result is not unexpected because there are other drugs than topoisomerase II inhibitors involved in our treatment protocols.

The synthesis of MT by tumor cells was proposed as a possible mechanism for the intracellular inactivation of drugs. Farnworth et al. [37] showed increased levels of MT in a cisplatin-resistant L 1210 mouse leukemic cell line. Kelley et al. [9] found that a human carcinoma cell line with overexpression of MT was resistant not only to cisplatin, a drug which is not involved in ALL therapy, but also to alkylating agents and anthracyclines. In our investigation only a tendency for an unfavorable prognosis was seen for patients with MT expression and the differences were statistically not significant.

Methotrexate-resistant cells isolated from clinical samples usually have elevated DHFR activity. The patients

of our investigation were treated with high doses of methotrexate and so we also analyzed DHFR. However, we did not find differences in disease-free survival of patients with DHFR-negative and DHFR-positive ALL. We have to take into account that very high doses of methotrexate were used in our treatment protocol and that the high concentrations might be able to circumvent methotrexate resistance in spite of the presence of DHFR proteins.

The intrinsic levels of TS have been shown to correlate with the resistance to 5-fluorouracil, but tumor cells resistant to other drugs also display increased levels of TS [11]. Scanlon and Kashani-Sabel [38] analyzed the activity of TS in a human ovarian cell line resistant to cisplatin and these resistant cells also exhibited an increase of TS. Although the proteins increase during chemotherapy, we found no differences in relapse-free intervals in patients with TS-negative and TS-positive leukemia. Our data suggest that only P-glycoprotein, glutathione S-transferase-π and, within some limitations, metallothionein had prognostic significance in ALL, whereas no relationship exists between the expression of topoisomerase II, dihydrofolate-reductase and thymidylate-synthase.

Our results also provide evidence that resistance is frequently multifactorial and there is evidence that a coexpression exists between several resistance-related proteins [25,39]. This coexpression may be regulated by proto-oncogenes and indeed a complex of c-fos/c-jun protein binds specifically to AP-1 site, which is present in genes that code for P-glycoprotein, glutathione S-transferase and metallothionein. In fact, in this investigation we found that the relapse-free intervals were significantly shorter in patients with Fos-negative ALL than in patients with Fos-positive ALL.

A possible involvement of second messenger systems and their related enzymes in the development of resistance has been discussed [40,41]. In current studies we found an increased expression of PKC in untreated primary cultures of renal cell carcinomas. This expression was significantly correlated with resistance to doxorubicin [42]. Furthermore, we analyzed the expression of PKC in nonsmall cell lung carcinomas and found a significant interrelationship between PKC expression and resistance [43]. In the present investigation, it was also revealed that expression of PKC is, in addition to the peripheral blast cell count, a significant prognostic factor for the clinical outcome after chemotherapy.

Out of a total of 10 factors screened for prognostic power for event-free survival, the four factors PKC, Fos, GST and P-170 were statistically significant at the level of $P = 0.05$. When we take into account the multiplicity of testing and reduce the significance level according to the Bonferroni adjustment to $0.05/10 = 0.005$, only PKC and Fos remain formally significant and GST and P-170 are subject to some degree to the possibility of having appeared by chance only. Nevertheless, their borderline significance with a *P*-value less than 0.03 and having clearly some prognostic value compared to the other six factors justify their consideration for prognosis and further investigation.

The presence of the resistance-related proteins Fos, PKC, P-170 and GST-π was not independent within our patient population. The analysis shows that in combination with PBC and PKC, both GST and P-170 have no separate prognostic influence. A further multivariate analysis synthesizing the factors PKC, Fos, GST-π as a categorical variable showed that this variable is even a stronger prognostic factor than the blast cell count, when dichotomized at 50,000 cells/mm^3.

Prognostic factors in cancer serve many purposes: they are used to understand the natural history of cancer, to identify homogeneous patient populations, to characterize subsets of patients with unfavorable outcome, to predict the success of therapy and to plan follow-up strategies [44,45]. In this investigation, the prognostic significance was limited to the clinical outcome after cytostatic treatment (relapse-free interval). We found that beside the very important blast cell count, other cellular parameters are additional prognostic factors for the clinical outcome of children with ALL. These additional prognostic factors are available at acceptable costs.

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